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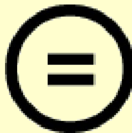

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A THESIS FOR THE DEGREE OF MASTER OF SCIENCE

THE CONSTRUCTION OF FUNCTIONAL GENE VECTOR FOR BIOREACTOR AND ESTABLISMENT OF EFFICIENT TRANSFECTION IN MAMMALIAN CELLS

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Department of Applied Biotechnology

GRADUATE SCHOOL

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June, 2013

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Do Luong Huynh

(Supervised by Professor Dong-Kee Jeong)

A Thesis submitted in partial fulfillment of the requirement for the degree of Master of Applied Biotechnology

2013.06

This thesis has been examined and approved by

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요 약

항균성 펩타이드 (AMPs)는 모든 종들 사이의 진화로부터 보존된 입자이다. 이 펩타이드는 항생제의 광범위한 스트럼에 대해 잠재적이며 항상제의 강한 저항력에 맞서는 새로운 치료제이다. 천연항생물질로서 AMPs 는 그람 음성 및 양성 박테리아, 바이러스, 곰팡이, 심지어 변형된 암세포를 죽이는 능력을 가지고 있다. 그뿐만 아니라 AMPs 는 면역 체계를 강화하여 면역 조절제로서도 작용할 것이라고 생각된다.

락토페리신은 단백질 분해효소인 펩신에 의해 분해되고 항균성을 가지는 펩타이드로서 락토페린보다 높은 항균성을 가지며, 이온 결합 단백질이다. 현재, 이 펩타이드 생산을 목적으로 하는 연구가 진행되고 있으나 그 효율은 매우 낮은 편이다. 본 연구의 목적은 PiggyBac 벡터를 생쥐의 배아 섬유아세포 3T3-L1 시스템에 도입하여 락토페린의 효율적인 생산과 발현을 관찰하는 것이다. 락토페리신 B 의 발현은 역전사효소중합연쇄반응과 단백질 전기영동 실험을 통해 확인하였다. 또한 다른 분자생물학적 방법을 이용해 PiggyBac 시스템의 효과를 평가하였다. 락토페리신 B 는 부작용 없는 항균치료에 도움이 될 것이며, 항균단백질의 생산 및 식품의 저장, 생물반응장치 시스템과 관련하여 새로운 접근법을 제시 할 것이다.

I. INTRODUCTION

1.1 Antimicrobial peptides

Antimicrobial peptides (AMPs) are small fragments evolutionarily belonging to innate immune system. These peptides have a potential function in antibacterial, fungi, enveloped viruses, and some cancers (Giuliani *et al.* 2007). Antimicrobial peptides can disrupt cell membrane or intervene the metabolism of microorganism, or target on cytoplasmic units by interaction between cationic targets of AMPs and anionic AMPs. Complementing to conventional antibiotics, AMPs have a broad-strum range in antimicrobial activities and less time of effects (Table 1).

Due to the surface of bacteria membrane is more negatively charged than mammalian cells, AMPs have a high affinity to bacteria, leading to selectively target on bacteria only (Giuliani *et al.* 2007). Furthermore, there are cholesterol molecules outside of mammalian cell's membrane as well as trans-membrane potential which have believed to reduce AMPs effects and partly contribute to the selective attack of AMPs on bacteria. Therefore, more than ever, it is necessary to study more to figure out the new AMPs, new role, new targets and taking full advantage of innate immune system for a particular treatment both in animals and human.

Table 1: Antimicrobial peptides (AMPs) with broad range against to microorganism. (Clare and Swaisgood 2000).

Milk peptide fragment	Release Protease	Gram + activity	Gram – activity	Yeast and fungi	Reference
Casecidin α_{s1} and κ-CN (MW=4000-6000)	Chymosin and chymotrypsin	Staphylococcus Sarcina Bacillus subtilis Diplococcus pneumonia Stepococcus pyogenes			Lahov & Regelson 1996
Casocidin-I α_{s2}-CN (f 165-203)	Synthetic peptide	Staphylococcus carnosus	Escherichia coli		Zutt *et al*. 1995
Isracidin α_{s1}-CN (f1-23)	Chymosin and chymotrypsin	Staphylococcu aureus		Candida albicans	Lahov & Regelson 1996
Lactoferricin B Lactoferrin (f17-41)	Pepsin	Bacillus Listeria Streptococci Staphylococci	E.coli 0111 E.coli 0157:H7 Klebsiella Proteus Pseudomonas Salmonella	Candida albicans Dermatophytes: Cryptococcus Uniguttulatus Penicilum pinophilum Trichophyton mentagrophytes	Bellamy *et al*. 1994, Shin *et al*. 1998, Tomita *et al*. 1991

Some of AMPs are naturally induced and target on both negative-gram and positive-gram bacteria with the same effects on conventional antibiotics without concerning about antibiotic resistance.

1.2 Bovine lactoferrin

Bovine milk has long been considered as a drinking to maintain health. Thesedays, many reports claimed that bovine milk and derives take a necessary role not only maintenance of health, but also have other functions (Noreddine bekerroum 2010). One of such properties is antibacterial activity that would be a promising candidate to replace conventional therapy using antibiotics, which has been thought relating to severe antibiotics resistance.

Lactoferrin, one of types of functional genes has been found in bovine milk (Adlerova *et al*. 2008). Lactoferrin naturally participates in antibacterial, antiviral, anticancer due to two mechanisms related to its positive charge (Figure 1). Firstly, as a member of non-haem iron-binding proteins, Lactoferrin can uptake iron which has been thought to halt the growth of bacteria. Second mechanism is that positive charging amino acids of Lactoferrin are able to directly neutralize outer negative charging amino acids of surfaces of bacteria, fungus, virus, parasites. Accordingly, cell membrane was lysed, leading to the inhibition of microorganism growth (Figure 2).

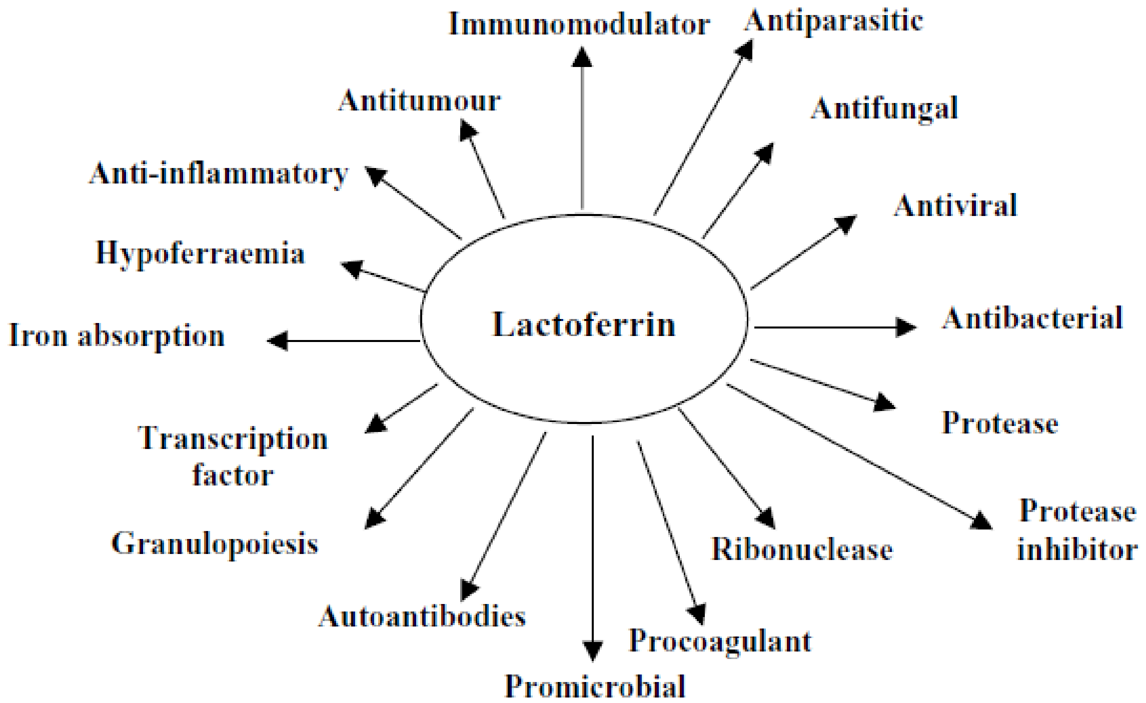


Figure 1: **Multirole of Lactoferrin proposed. (Farnaud & Evans 2003)**

Existing as a member of transferrin, Lactoferrin can uptake iron which is necessary for bacteria growth. Therefore, lactoferrin partly inhibits the growth of bacteria. Furthermore, not only taking role in antitumor, antiviral, antibacterial, antifungal, Lactoferrin also owns other special properties enhancing immune system activity.

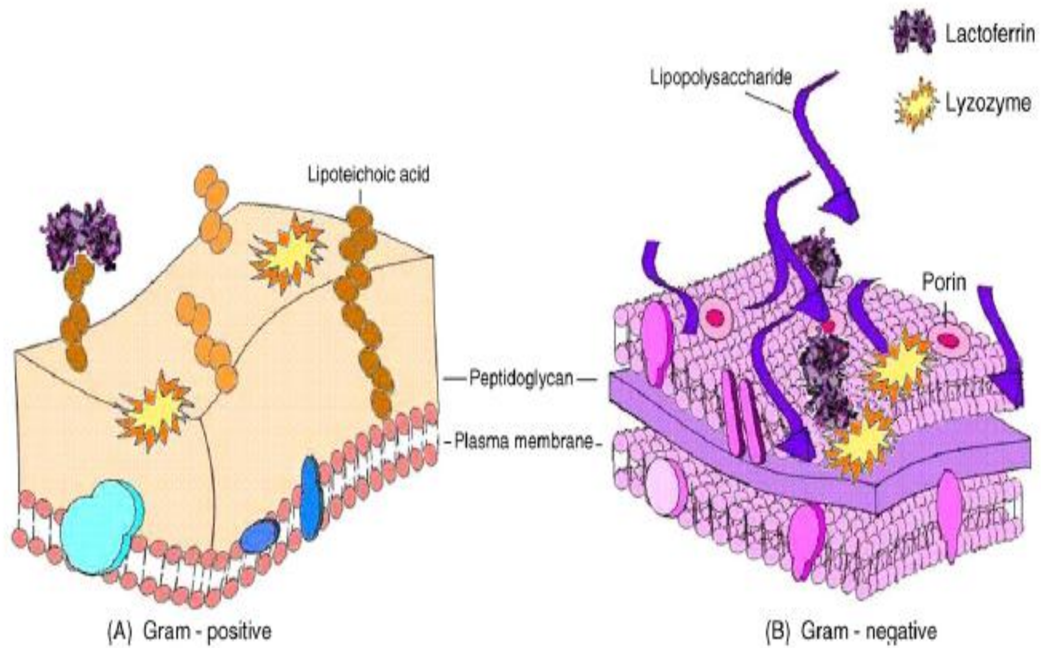


Figure 2: Mechanism of Lactoferrin targeting on cell membrane, causing cell lysis.

In gram positive bacteria, Lactoferrin can bind to molecules negatively charging (ex Lipoteichoic acid) due to its positive charge, facilitating the cell membrane lysis (A). In gram negative bacteria (B), Lactoferrin directly interacts on Lipopolysaccharide layer, followed by enhancing the cell lysis progress. (Susana A. Gonzalez Chavez *et al*. 2009)

1.3 Bovine Lactoferricin

Generated by digestion of Pepsine, Lactoferricin is a small fragment of Lactoferrin with the length 25 amino acids (Figure 3), has multirole in anti-microbial and anti-cancers (Farnaud & Evans 2003).

Although Lactoferricin has no relation to iron catching, many evidences support a fact that Bovine Lactoferricin (LfcinB) has strong ability in antimicrobials, even better than original one (Marr *et al*. 2009, Gifforda *et al.* 2005) (Figure 4). Due to be existed naturally in body as uptake bovine milk, the effects of lactoferricin have promising as a new novel therapeutic therapy in attempt to cure severe disorders in digestion system, for instance diarrhea, and could be an adjuvant in treatment of cancers without side effects (Gifforda *et al*. 2005, Mader *et al*. 2005) as well as in preservation of foods (Touch *et al.* 2009). Therefore, it is necessary to produce more this bioactive peptide from mammalian system for further studies and clinical trials.

Thus, in this study, we synthesized a fragment from 17-41 amino acids to investigate ability of LfcinB production in the mammalian cell line system and heading to produce in *in-vivo.*

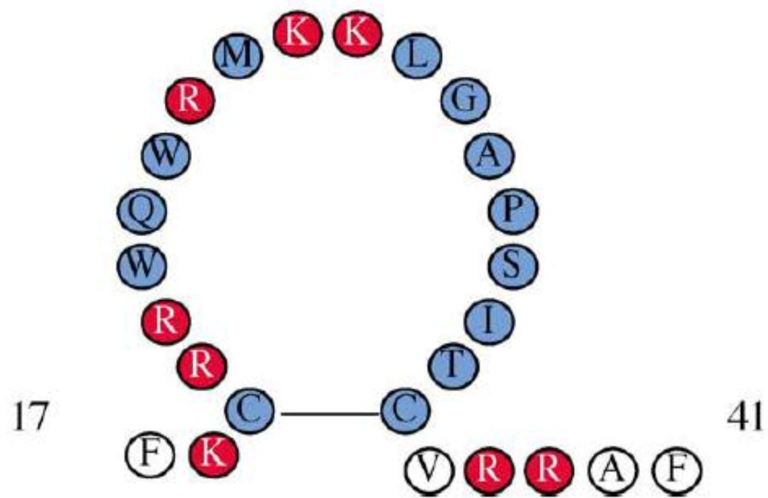


Figure 3: Structure of Bovine Lactoferricin. (Farnaud & Evan 2003)

Bovine Lactoferricin, a fragment of Lactoferrin located from 17-41 amino acids, contains loop which get involved in antibacterial activity.

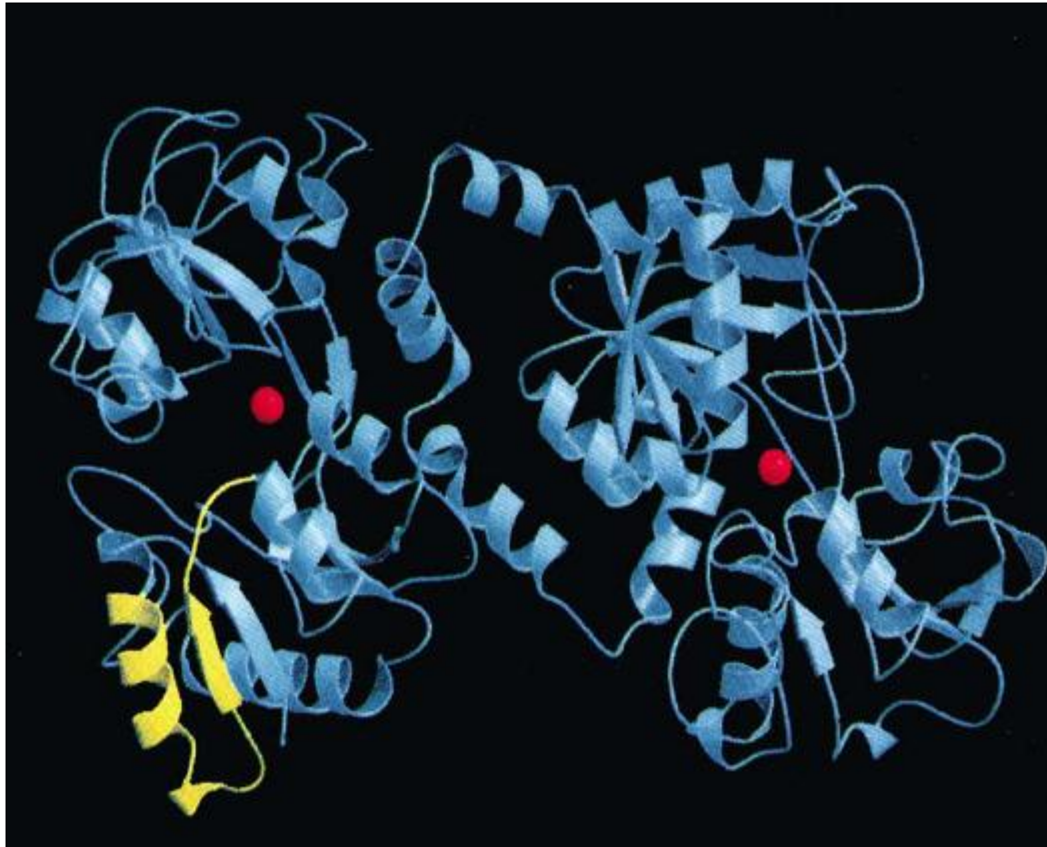


Figure 4: Lactoferrin and Lactoferricin in 3D structure. (Farnaud & Evans 2003)

Lactoferricin (yellow) has no relation to catching iron haem part of lactoferrin (red). Not by inhibiting growth of bacteria by catching iron, some evidences, nonetheless, demonstrated that Lactoferricin has a stronger impact in anti-microbial activity than Lactoferrin (blue),

1.4 Piggybac expression system

PiggyBac (PB) transposon has been found as a genetic mobile element acting to transpose from vector into DNA genome through "cut and paste" (Figure 5). Through the activation of PB transposase (Figure 7), inverted terminal repeat sequences (ITRs) flanking at both ends target gene were recognized and efficiently integrates the contents from the original sites and efficiently integrates into TTAA chromosomal sites. Therefore, once expressed by PiggyBac, the expression of target gene would be strong and steadily for long term (Cadinanos & Bradley 2007, Ding *et al*. 2005, Li *et al.* 2011, Mitra R. *et al.* 2008, Owens *et al.* 2012, Wu *et al.* 2006, Nakanishi *et al.* 2010, Doherty *et al*. 2012).

Linking to the need expressing efficiently, 3T3-L1 mouse embryonic fibroblast – adipocyte like cell line is considered as one of cell lines suitable for production of protein. Indeed, 3T3-L1 has been long time used to examine in the production of cytokines and effects of exogenous factors on the other gene expression (Mater et al. 1998, Cheng et al. 2006, Chang et al. 2012). Thus, it might be that 3T3-L1 system could be an ideal model for expression of target gene.

With the new approaching in production of Bovine Lactoferricin, PiggyBac vector will promise a new facility to firstly examine the expression of Bovine Lactoferricin in mouse embryonic cells and secondly to estimate, whereby, ability of production of Bovine Lactoferrin *in vivo*.

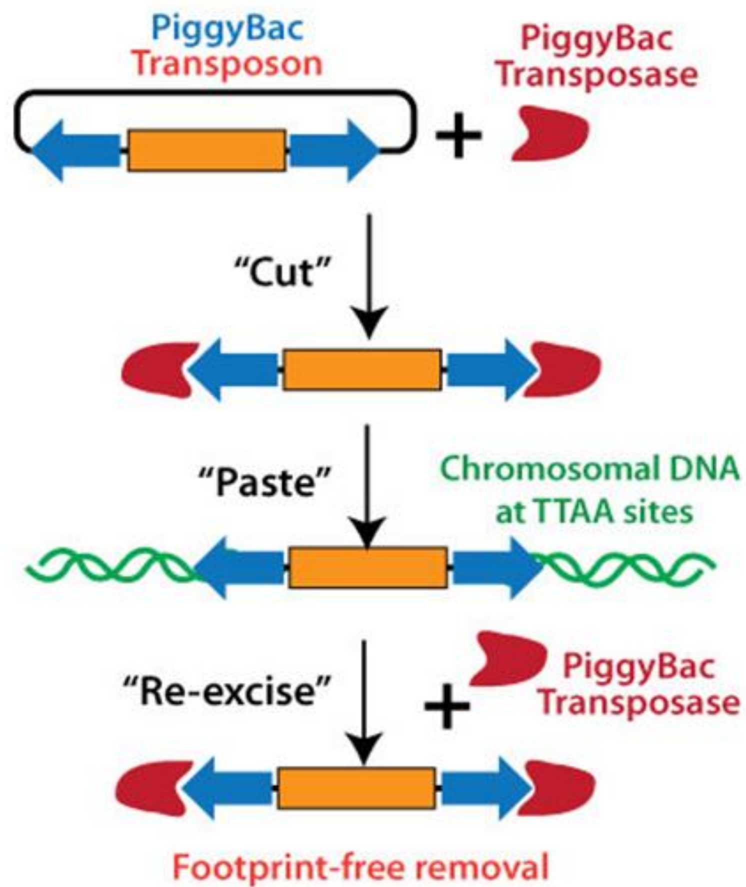


Figure 5: Mechanism of transposition in PiggyBac system.

Flanking with reverted elements which are recognized by PiggyBac transposase, target gene is integrated into DNA chromosomes by mechanism of "Cut and Paste" specific at TTAA sites. For the remove of modification of chromosomes, PiggyBac transposase can be supplemented for footprint-free removal.

II. MATERIALS AND METHODS

2.1 Reagents

DMEM/F12 medium, fetal bovine serum (FBS), antibiotic-antimycotic, Trypsin/EDTA 0.5%, Superscript II first strand cDNA synthesis kit and are all purchased from Gibco. TritonX-100, human epithelial growth factor (hEGF), PMSF, were original from Sigma Chemical Co. (St.Louis, MO). Primary antibody Bovine Lactoferricin is from Genway Biotech, Inc. secondary antibody conjugated with phycoerythrin (PE) was purchased from eBioscience, US. Ex Taq polymerase, enzyme restriction EcoRI and BamHI were ordered from Takara, Japan. Enzyme Ligase is from Invitrogen. Gel purfification kit is made by Bioneer Co., Korea. Total easy Blue RNA extraction kit and plasmid extraction, E.coli Top10 are from IntronBiotech, Korea. Maxi-Prep plasmid extraction kit is original from Quiagen. Pierce BCA protein assay kit was purchased from Thermo scientific, US. Plasmid PiggyBac and PiggyBac helper were purchased from System Bioscences (SBI). Bovine lactoferricin was synthesized and cloned in plasmid pGEM-B1 (Bioneer). Puromicin and Ampicilin were purchased from GeorgiaChem, Co. Korea. Amaxa Nucleofection kit and plasmid pmax GFP was purchased from Lonza, Co. Evagreen dye was purchased from Biotium, Co. US.

2. 2 Cell culture

Mouse embryonic fibroblast – adipocyte like cell line (3T3 L1) was cultured in DMEM/F12 supplemented with heat-inactivated 10% FBS, 1% antibiotic-antimycotic and 10ng/ml human epidermal growth factor (hEGF). At 80% of confluence, cells were subcultured.

2.3 Plasmid construction

Fragment encoding for Bovine Lactoferricin (LfcinB) was picked up from plasmid pGEM-B1 containing LfcinB by Polymerase chain reaction (PCR) with left Primer ATA GAT CTG ATA TCG CTA GCG AAT TCA AGC and right primer TAG GAT CCG ATA TCG GTA CCG CCG GGC TCG AGC C. Thermal cycling parameter were 95^0C 5min, 95^0C 30sec, 67^0C 30sec, 72^0C 1min, cycle was repeated 35 cycles, finally 72^0C 10min and hold at 4^0C. PCR products were separated on gel agarose 1% and extracted by Gel purification kit. PCR products, Bovine Lactoferricin (LfcinB) flanking with bovine signal peptide and bovine growth hormone polyA was clone into mammalian expression Vector PiggyBac (PB) by enzyme restriction EcoRI and BamHI as manufacturer's instruction (Figure 6). Ligated products were transformed into E.coli Top10 with 100ug/ml Ampicillin selection marker. PCR colonies screening was conducted, followed by double digestion and PCR with specific primers to confirm recombinant vector. Finally, fragment containing LfcinB was sequenced by Bioneer Co. Korea.

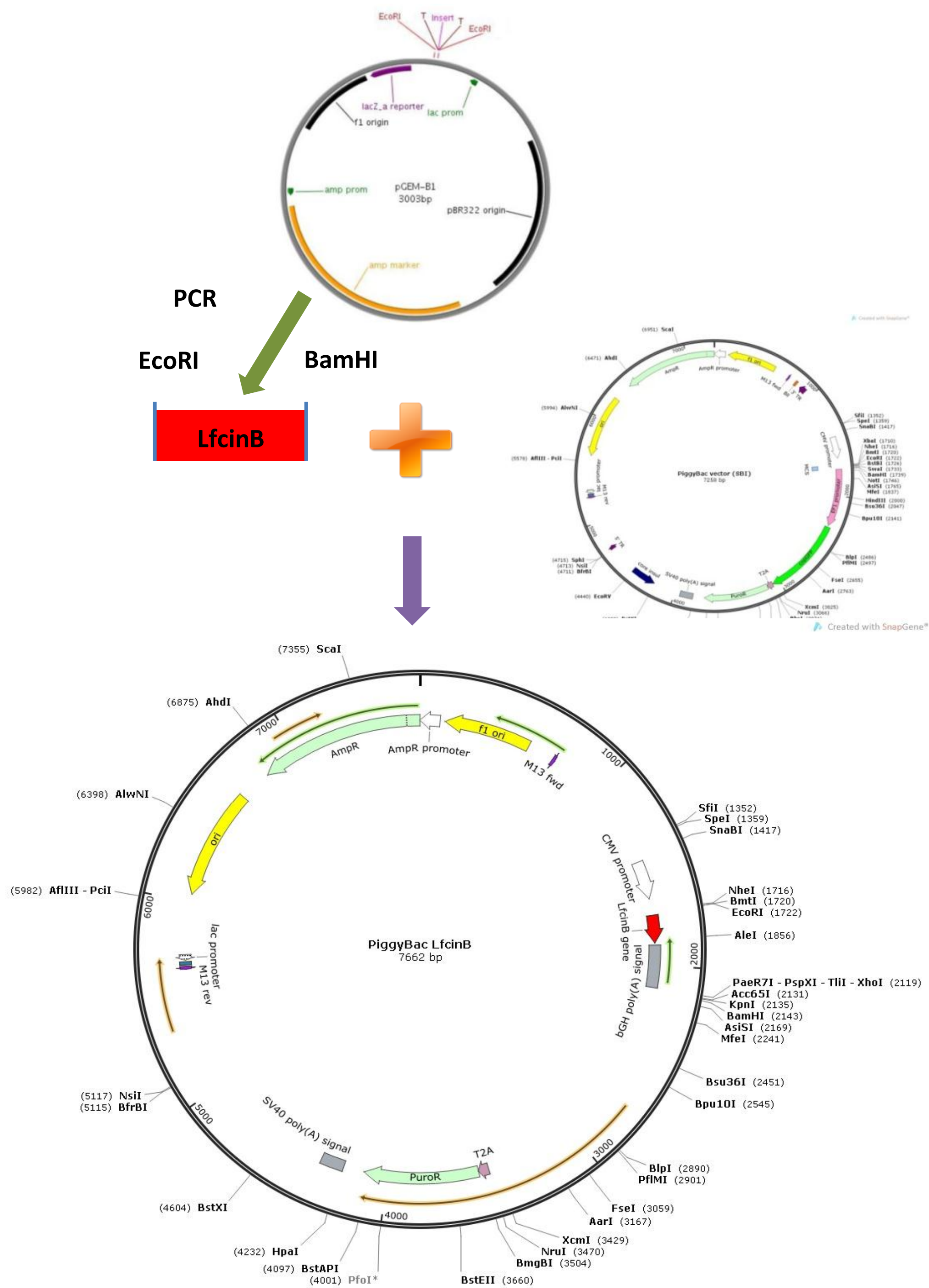


Figure 6: **Construction of PiggyBac**

Lactoferricin Bovine has been picked up by PCR with primers ending with EcoRI and BamHI, followed by cloning into PiggyBac vector.

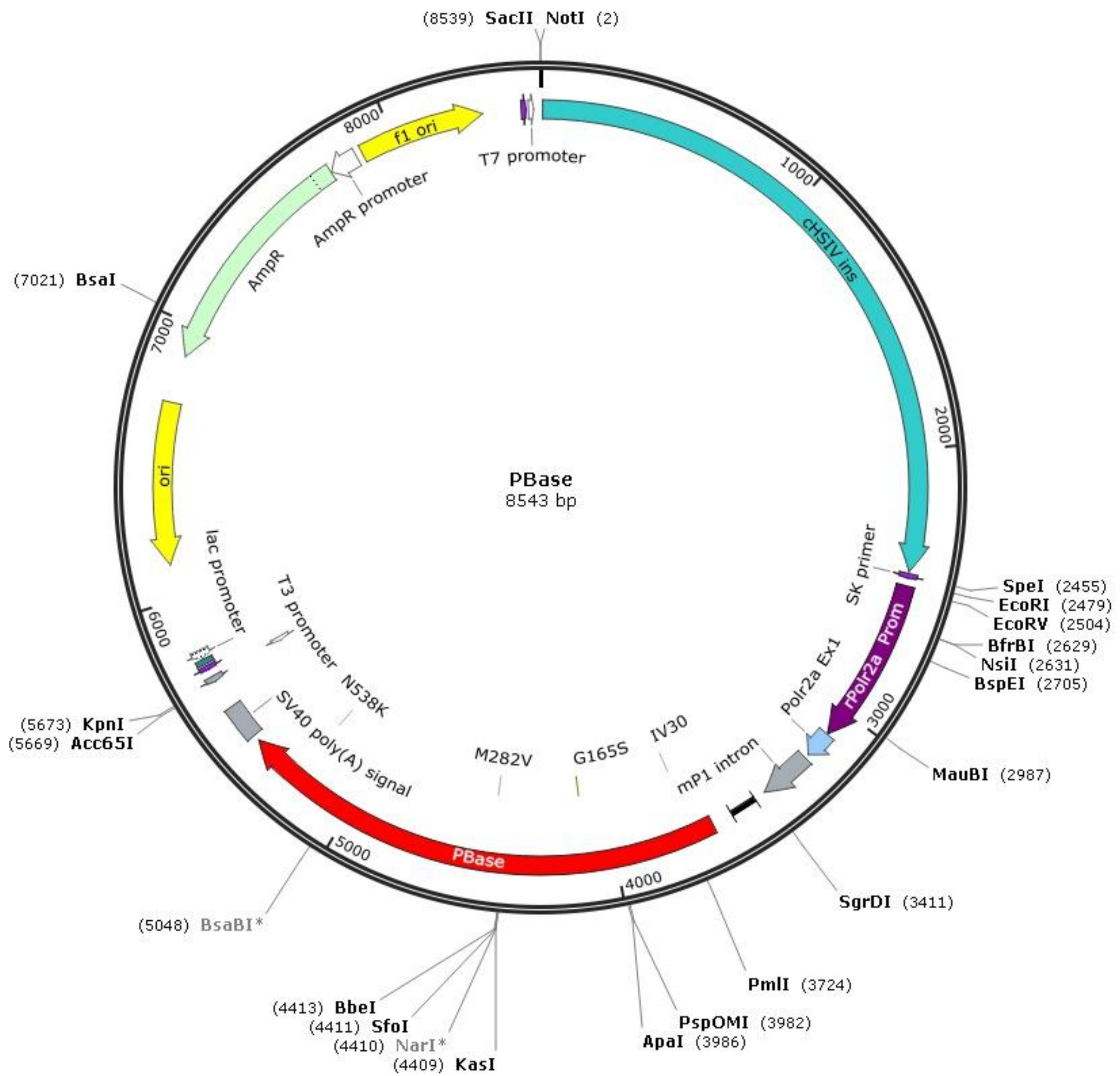


Figure 7: Structure of Vector PBase helper

Vector PBase encoding the enzyme transposase which takes a role in integration of target gene at TTAA sites of DNA genome.

2.4 Cell transfection

2.5 ug PiggyBac contanining Bovine Lactoferricin after removed endotoxin by Triton-X114 method were used for transfection. Trypsinized 3T3-L1 Cells were mixed with the mixture of helper PBase vector (Figure 5) and PiggyBac-vector containing Bovine Lactoferricin with the ratio 1:1 respectively, or 5 ug pmax Green Flourescent protein (GFP) vector (Lonza, Amaxa) containing GFP gene as positive control and nucleofected by A23 program of AMAXA electroporation system. After 24hrs, cells were examined on fluorescent microscope.

2.5 Selection of stable cell line

48hrs post-transfection, 3T3-L1 cells were applied for selection with 1ug/ml Puromicin. During 2 weeks, colony of cells detectable with GFP was picked up by diluted trypsin method in order to obtain the homologous population of GFP-expressing Cells. Colonies formed by 3T3-L1 cells were continuously selected by 1ug/ml Puromicin for further analysis.

2.6 RT-PCR

5ug total RNA of transfected cells was extracted by total easy RNA extraction kit (INTRON biotech) used as template for reverse transcription via superscript II first strand cDNA synthesis kit. cDNAs, afterward would be used for PCR to detect expression of interest gene, Bovine Lactoferricin. Primers to PCR cDNA from mRNA LfcinB are CCCTGCTGTCCCTTGGAG and TAAAAGGCCCTCCTCACACA, respectively. Pair of mouse beta-actin primers including left primer ACTGGGACGACATGGAGAAG and right primer GGGGTGTTGAAGGTCTCAAA were used as internal control as compared to Bovine Lactoferricin primers at $T_{annealing} = 63^0C$.

2.7 Relative quantification

cDNA from 3T3-L1 nontransfected and transfected were used for real-time PCR with Evagreen dye (Biotium) to examine the relative expression. Mouse beta actin was utilized as internal control. The left primer is TTGGACTGTGTCTGGCTTTC and the right primer is GCGGCCGCTTATCACTAAA, respectively. Via the emission of Evagreen, the concentration of Bovine Lactoferricin was examined as compared with mouse beta-actin.

2.8 Immunocytochemistry

Transfectants were seeded on 4-well plate, at 50% confluence. Before blocking with goat serum for 10minutes, cells were fixed in 4% paraformaldehyte within 30min at 4^0C, treating with 0.2% Triton X-100 for 30min at 4^0C, followed by blocking endogenous peroxidase with 0.3% H_2O_2 for 10min. For blocking non-specific binding, cells were incubated with 10% goat serum in 4^0C at 1 hr. Washing 3 times with PBS, immunostaining with primary antibodies specific to Bovine Lactoferricin original from mouse was supplemented at dilution 1:5 overnight at 4^0C in dark humidity area. For visualization, secondary antibodies goat anti mouse conjugated with Phyoerin (PE) was added at dilution 1:50. To visualize nuclei of cells, cells was couterstained with 4,6-diamidino-2-phenylindole (DAPI) (Sigma) at 0.1µg/mL in 20min. Immunostained cells were observed on a fluorescent microscopy (Olympus).

2.9 Tricine SDS PAGE analysis.

Due to Bovine lactoferricin is a small peptide, approximately 3.1kDa, it is impossible to run SDS-PAGE as usual. Thus, Tricine SDS PAGE is a suitable method utilized, which has been considered applying for small peptides and proteins. Briefly, as mentioned in Hermann Schagger 2006, total proteins were extracted by RIPA buffer, measured concentration by Pierce BCA kit, and loaded in to stacking gel 10% acrylamide while 16% gel acrylamide is used for running gel. Initial voltage is 30V, next is 200V and finally is 300V. For protein visualization, proteins were stained with Coomassie Blue and detected with target band ranged between 2-5 kDa.

III. RESULTS

3.1 Plasmid construction

Recombinant plasmid after PCR contains target gene at 449 bps as compared to positive control – donor plasmid GEM-B1. That demonstrated partly recombinant plasmid is concerning plasmid necessary for further study (Figure 8).

Next, concerning recombinant plasmids are ready for single and double digestion of EcoRI and BamHI. Results showed raising the band of interest at 449 bps at double digestion and at 7762 bps for single digestion (Figure 9). That demonstrated that plasmid concerning contains target gene, Lactoferricin from Bovine.

Afterward, plasmids were sequenced for final confirmation.

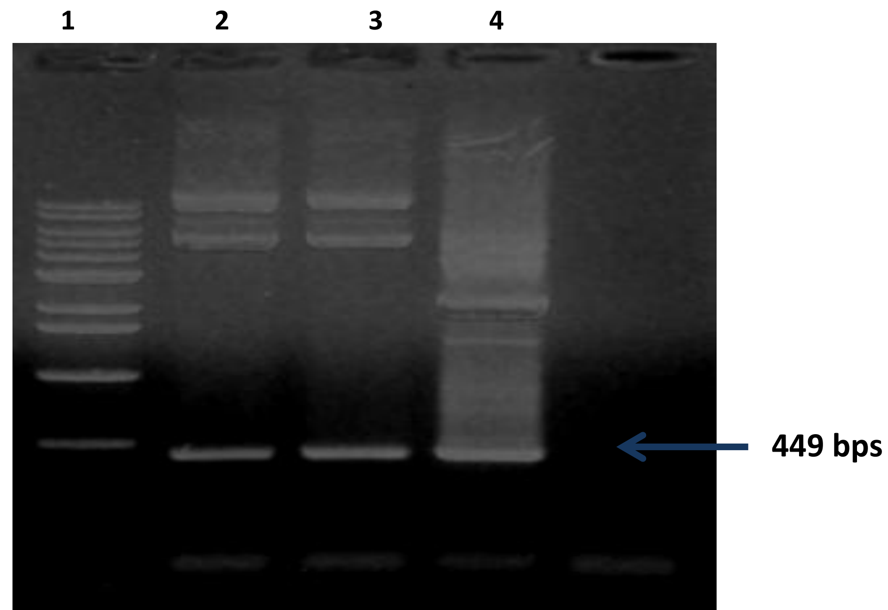


Figure 8: PCR recombinant vector after cloning.

Lane 1: Ladder 1kd Bioneer; Lane 2, 3: recombinant plasmid; Lane 4: plasmid GEM-B1 containing Lactoferricin Bovine.

Plasmids concerning after PCR showed the same band with positive control at 449 bps, that left a fact that plasmid concerning has target band, Lactoferricin from bovine.

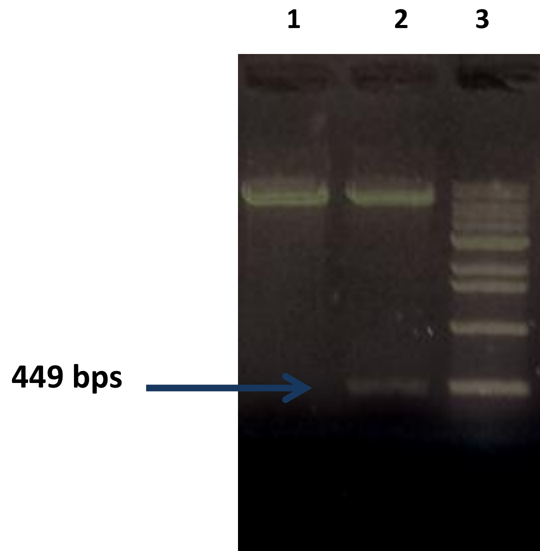


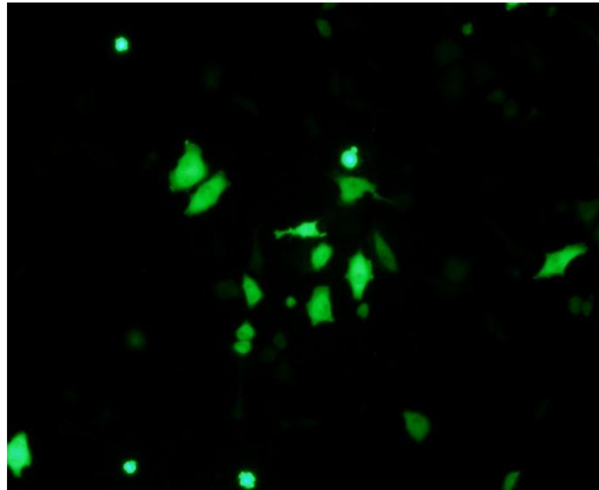
Figure 9: recombinant plasmids in digestion

Lane 1: single digestion with EcoRI; Lane 2: double digestion with EcoRI and BamHI, Lane 3: 1kb ladder.

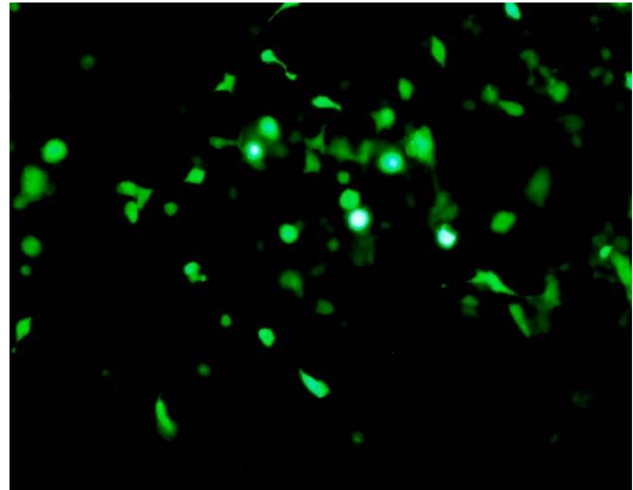
For the confirmation, concerning plasmids were digested single or double. To single digestion result, the band showed at 7762 bps, corresponding to predicted recombinant plasmid. Moreover, with double digestion, the result showed the band with 449 bps representing for target gene cloned. Thus, it is believed that concerning plasmid is recombinant plasmid and available for further studies.

3.2 Cells transfection

The mixture of 2.5 ug PiggyBac and 2.5 ug PBase with the ratio 1:1 has been applied for transfection. The result showed the expression of Green fluorescent protein (GFP) after 48hrs even effectively as compared to positive control (Figure 10). Primarily, it is definitely that the utilization of PiggyBac system is more sufficiently than normal vector. That partly demonstrated that Piggybac is one of powerful tool for transfection.



Pmax GFP



PiggyBac-LfcinB

Figure 10: 3T3-L1 tranfsfected (10X) with PiggyBac - LfcinB by using Amaxa protocol. After 24hrs, transfected cells showed the expression of Green Flourescent Protein (GFP) in both positive control (pmax GFP, Lonza) and PiggyBac-LfcinB. It seems to be that ratio PiggyBac/ PBase Helper 1:1 in transfection is a good mixture as compare with positive control.

3.3 Selection of colony

During time of selection, non-transfected 3T3-L1 without ability resistant to puromycin (1ug/ml) would be detached. Gradually, transfectants have a chance to grow and form colony surrounding original place (Figure 11).

Colony has been trypsinized with very diluted concentration of Trypsin and pick up purified colonies to transfer to fresh medium for multiplication. After 14 days for selection, colony was expanded and multiplied as before transfection (Figure 12)

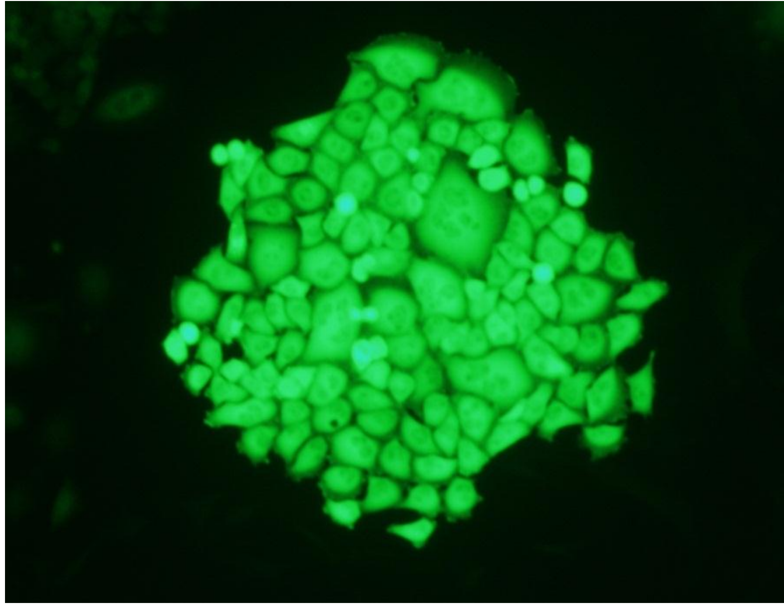


Figure 11: colony of 3T3-L1 tranfsfected (20X) with PiggyBac containing LfcinB selected in 1ug/ml Puromycin

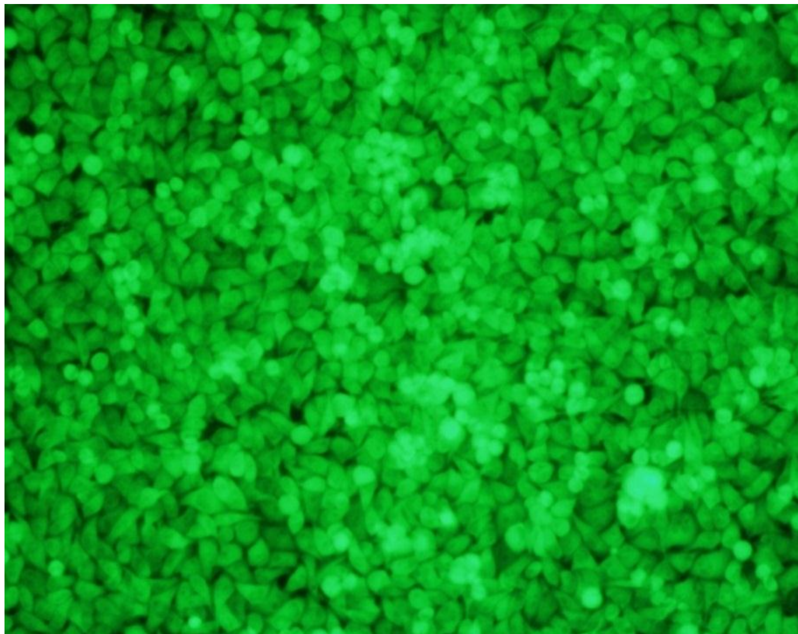


Figure 12: Colony after 2 weeks for selection in expandsion (20X)

3.4 RT-PCR

1ul of diluted cDNA (50ng) in both nons-transfected and transfected cells was used as template for second step in RT-PCR (Figure 13). After PCR, only targetband 115 bps in transfected cells raised in gel electrophoresis 1% as compared to non-transfected cells. Accordingly, for internal control beta-actin, 157 bps PCR product bands were representive for both nontransfected and transfected cells.

This clarified that mRNA of Lactoferricin bovine has been expressed properly while beta actin both group are equalized.

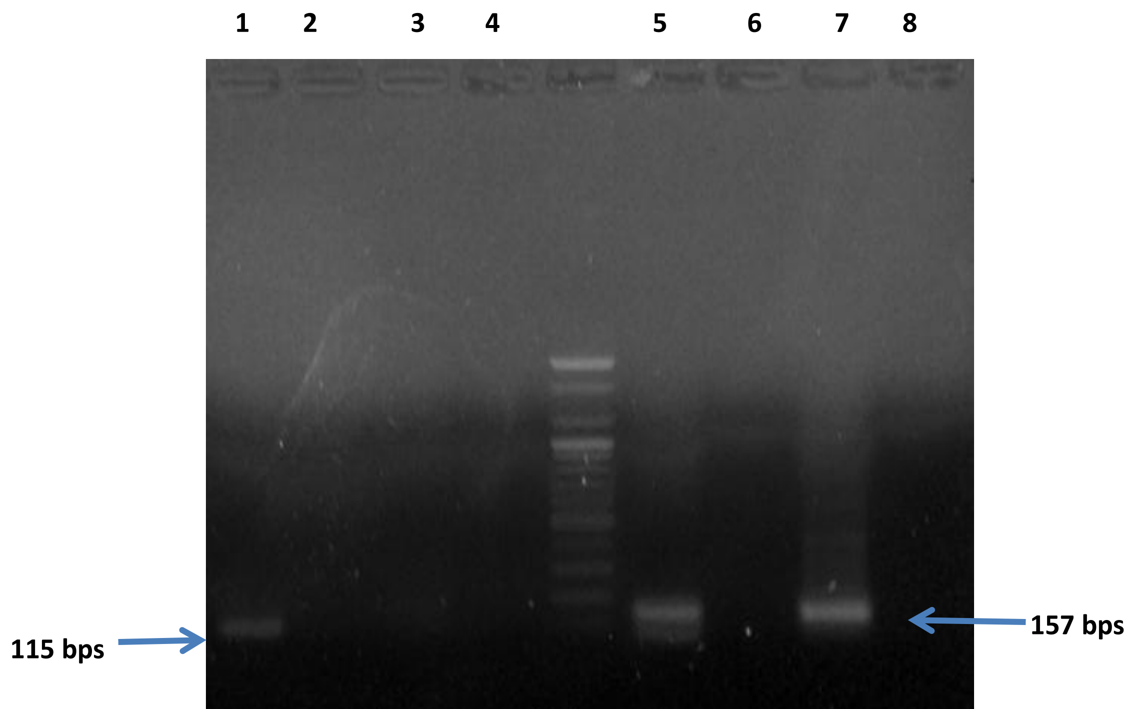


Figure 13: RT-PCR products from 3T3-L1 transfected and non-transfected cells.

Lane 1: cDNA from Transfected cell; Lane 2: negative; Lane 3: cDNA from non -transfected cells. Lane 4: negative of primers but adding cDNA . Lane 5 and 7 are cDNA from transfected cells and non-transfected cells with beta-actin primers. Lane 6 and 8 are negative for beta-actin. Ladder: 100bp (Bioneer)

Band of cDNA of transfected cells with 115 bps as compared no band in non-transfected group confirmed clearly just only transfected cells are positive with target band. Whereas the expression of beta-actin in both group are same.

3.5 Relative quantification.

After normalization, cDNA from both non-transfected and transfected 3T3-L1 cells were used as template for real-time PCR (Figure 14). By using Evagreen, the expression of target gene is significantly different between with 2 groups. For the transfected cells, there was expression of mRNA LfcinB. As a result, the signal for Evagreen is highly, approximately $10^{5.5}$ signal while in the column of non-transfected cells, there was no signal. That determined just only transfected cells expressing target gene with the enhancement effectively of PiggyBac. The internal control was utilized by mouse beta-actin to be as relative standard for examination.

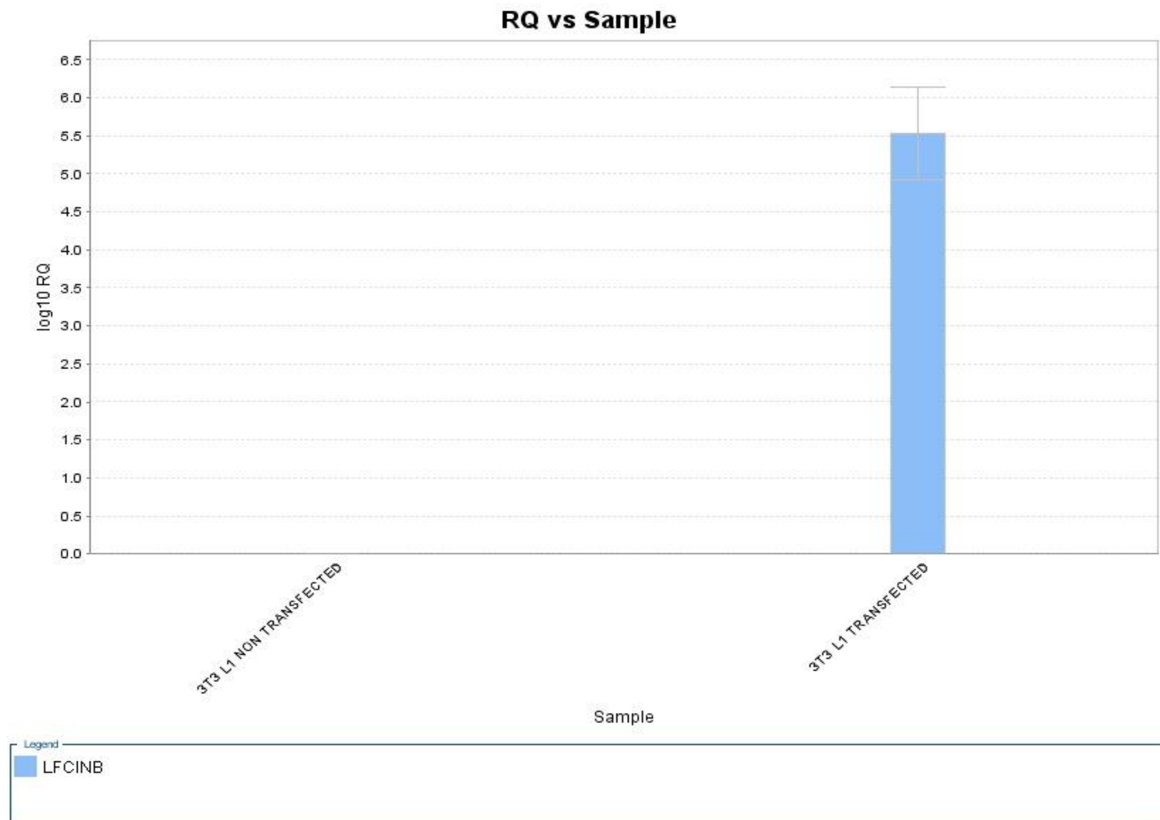


Figure 14: Relative quantification the expression in transfected cells. The 3T3-L1 transfected cells on the right are highly with signals from Evagreen as compared to the left, non-transfected cells. Mouse beta-actin was used as internal standard. All experiments were triplicated.

3.6 Immunocytochemistry staining

The 3T3-L1 cells transfected with PiggyBac-LfcinB was utilized for immunocytochemistry staining (Figure 15). In the negative control (A), transfectants with PiggyBac still showed the expression of GFP, without primary antibody, emission of Phyoerin are undetectable while the nucleus staining is encountered with DAPI. In the tested group (B), with the supplement of primary antibody, bovine lactoferricin antibody, transfected cells showed the red color emission as supplying with secondary antibody conjungated with Phyoerin while cells still were positive with GFP and cleared with fluorescence of DAPI. Results showed that cells transfected with PiggyBac have been expressing Lactoferricin bovine properly at translational level.

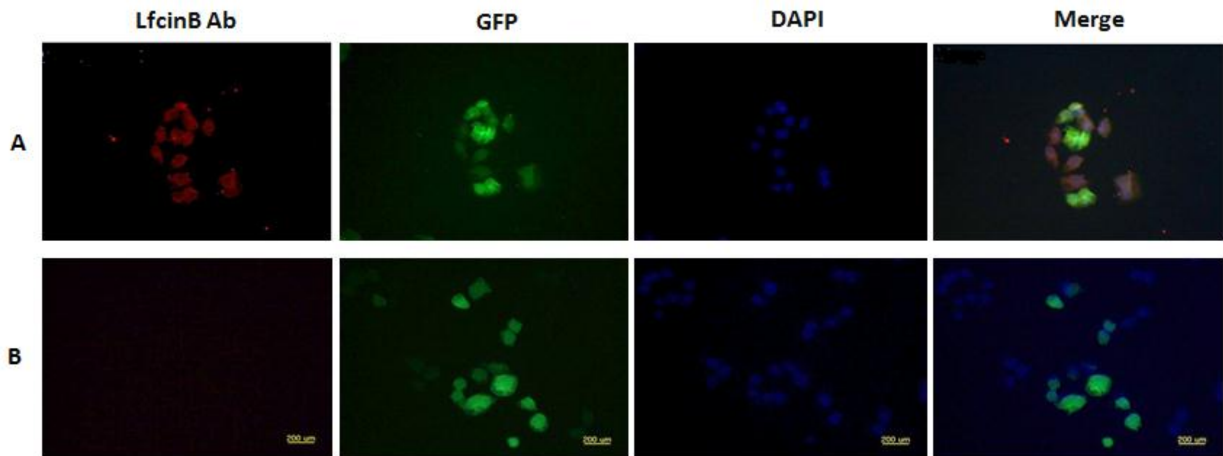


Figure 15: Immunostaining transfectants with LfcinB antibody. The transfected 3T3L-1 cells without primary antibody Lactoferricin Bovine, and with secondary antibody goat anti mouse secondary antibody conjungated with Phyoerin (PE). B: transfected 3T3-L1 immmunostained with mouse primary antibody anti Bovine Lactoferricin and goat anti mouse secondary antibody conjungated with Phyoerin (PE).

3.7 Tricine SDS-PAGE

18ug of total proteins were loaded to run SDS-PAGE. In the non-transfected cells, there was no band corresponding with 3.1 kDa, which is considered as band of lactoferricin bovine (on the left) (Figure 16). Whereas in remaining group, transfected cells had shown the expression of target gene with full of structure corresponding with the band of 3.1 kDa in SDS-PAGE (on the right). That means target gene-Lactoferricin Bovine has been expressed and formed correctly. Under SDS-PAGE, Lactoferricin was separated out of mixture and run to appropriate area as stimulation of electric field.

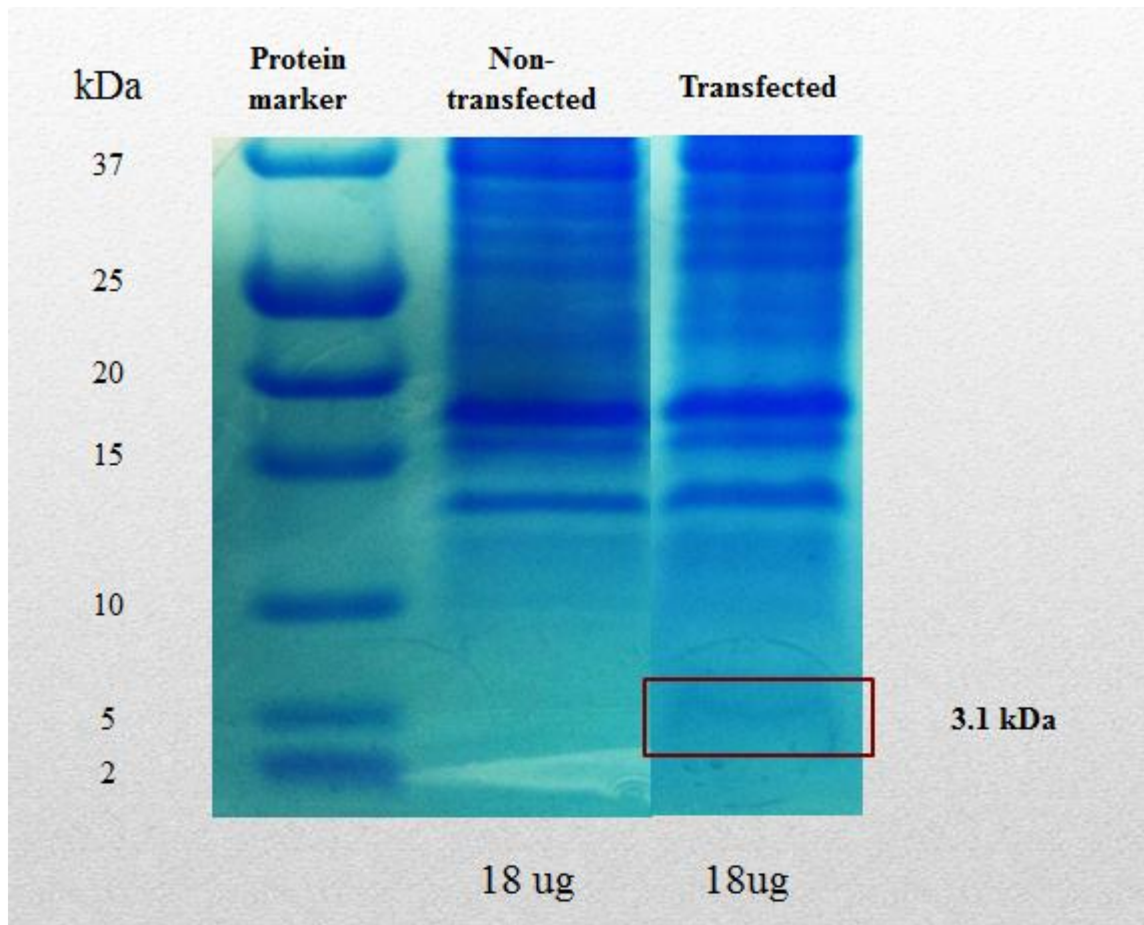


Figure 16: Tricine SDS-PAGE running with target band 3.1 kDa

Total proteins from both non-transfected and transfected cells were loaded for SDS PAGE running. In the transfected cells (right) showed the target band as compared to non-transfected cells (left). Tricine SDS-PAGE was run with 16% acrylamide.

IV. DISCUSSION

Lactoferricin has taken attention in science due to it possibility with multifunction in antibacterial, anticancer, antifungal, and parasites. Outstanding from such properties, antibacterial with no side effects has been considered as a new hope in naturally targeting to bacteria and as a replacement for conventional usage with antibiotics. Some studies have tried to produce Bovine Lactoferricin as Chen *et al*. 2009 in Pichia pastoris or Tian *et al.* in E.coli 2008. However, such methods owned some disadvantages such as peptides still contain endotoxin, a thing is a worry of most of recombinant products from microorganism system. Furthermore, such products are not produced on mammalian system, leading to trouble once applying on real clinical trials.

Other evidences also conducted the expression of human Lactoferricin in transgenic mice as Platenburg *et al*. 1994, or in HC11 mouse epithelial cell line by Myoung-Soo Nam, 2001. However, no evidence has been investigating on Bovine Lactoferricin. Therefore, in this study we have experimented to produce Bovine Lactoferricin in 3T3-L1 mouse embryonic fibroblast – adipocyte like cell, which is believed to response fast to stimulation as well as potent in in-vivo studies. Under the enhancement of PiggyBac system, vector considered as a power tool for transfection, target gene would be integrated into DNA chromosome for long term expression.

Indeed, PiggyBac containing Bovine Lactoferricin has showed the stable expression of GFP elongated to 30days of time surveyed without any selection (data not shown).

Under selection of 1ug/ul puromycin, transfected cells expressed strongly and started to form the pure GFP colonies during 7 days. On the next stage, within 14 days for selection, transfectants can expand and steadily 100% cells positive with GFP.

Next, purified transfectants were utilized for further studies. The results showed the expression of lactoferricin at RT-PCR and at translational level in positive with immunocytochemistry staining. These things demonstrated that transfection into 3T3-L1 cells is efficient with expression vector are working properly.

Furthermore, as compare with non-transfected cells, transfectants also were recorded the significant expression via real time relative quantification. Such things guaranteed that via the help of PiggyBac vector, Bovine Lactoferricin has expressed dramatically, an important term as transferring a new gene into strange host system. In overall, these data have been supporting a fact that Bovine lactoferricin can be expressed and produced in mouse embryonic cells. This would be a springboard to exams the impact of Bovine Lactoferricin on mouse *in-vivo* study in perspective.

V. SUMMARY

Antimicrobial peptides (AMPs) are conserved particles from evolution found among all classes of life. These peptides are potential to broad strum of antibiotics and probably are novel therapeutic agents before severe resistance of antibiotics nowadays. Working as natural defensing agents, AMPs has ability killing on both gram-negative and positive bacteria, virus, fungi and even transformed and cancer cells. Besides, it is believed that AMPs also works as immunomodulators by enhancing on immune system.

Lactoferricin (Lfcin), a small fragment of lactoferrin digested by pepsin has been demonstrated as AMPs owning the potential antimicrobial activity than lactoferrin, the native ion-binding protein representing in cow milk. Current studies have been conducted to produce this peptide, yet in mammalian system. Aim of this study was to produce effectively and examine the expression of Lactoferricin from Bovine (LfcinB) in the long-term by using PiggyBac vector transfected into mouse embryonic fibroblast adipocyte –like cells (3T3-L1) system. Under enhancement of PiggyBac vector, the expression of LfcinB was confirmed by reverse transcriptase polymerase chain reaction (RT-PCR), sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and other molecular methods for evaluating the effects of PiggyBac on the production of target peptide up-streamed with bovine signal peptide.

LfcinB with full of function would be helpful in anti-bacterial therapy naturally without side effects and provides a new approach in both the production of anti-microbial protein and preservation of foods, heading to bioreactor system.

Keywords: Bovine lactoferricin, antimicrobial, PiggyBac, Bioreactor.

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